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Existence of Zeatin, a Highly Active Cytokinin, in Blanching Water of Bamboo Shoots

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Abstract

Cytokinins contained in the blanching water of bamboo shoots were purified partially by *n*-butanol partition, ion-exchange Dowex 50W column, Ag precipitation, paper chromatography and Sephadex LH-20 column chromatography. Chromatographic behaviors of a cytokinin, which is extractable by water-saturated *n*-butanol from aqueous layer, held by Dowex 50W (H^+ form) and precipitated by Ag ions, suggest that the cytokinin is likely to be zeatin. From a comparison between the cytokinin activity in Ag precipitate and that of authentic zeatin it may be given as a conclusion that the blanching water of bamboo shoots contains zeatin approximately at 20 nM. Neither N^6-(Δ^2-isopentenyl)adenine nor its riboside was present in the blanching water of bamboo shoots.

Introduction

A number of N^6-substituted adenines, their ribosides and ribotides have been paid attention as cytokinins which have various physiological activities including the promotion of cell division in plant culture systems[1-3]. Moreover, it has been reported that many N^6-substituted adenines and some of their ribosides have antitumor activity in mammalian cell cultures[4-10]. Recently, zeatin, one of the strongest cytokinins, has been shown to have some regulatory roles in DNA replication of mammalian cells[11)12)].

On the other hand, cytokinins have been reported to be existent in many species of edible plants such as sweet corn kernels[13)14)], pea[15)], fruitlets of apple[16)17)], quince, peach, plum, pear, pumpkin, marrow[16)] and persimon[18)] and turnip root[19)]. Certain cytokinins have been also found in tRNA of some microbial, animal and plant species[20-25)], and identified as minor bese to locate next to the anticodon of specific tRNA species[20)22)24)]. These reports suggest that cytokinins present in edible plants have any significant roles also in some physiological processes of human being.

In a preliminary study on natural cytokinins it was found that an extract of bamboo shoots exhibited a strong cytokinin activity in the tobacco callus bioassay. On the basis of this finding, an attempt to purify cytokinins contained in the blanching water of bamboo shoots was carried out and revealed that the blanching water contained several kinds of cytokinins, a water-soluble cytokinin among which was likely to be zeatin ribotide being contained approximately at 20 mM[26)]. The present study reports partial purification and characterization of remaining, so to speak, water-unsoluble cytokinins in the blanching water of bamboo shoots.

Materials and Methods

The blanching water of bamboo shoots used in this study was obtained from Kakimoto Cannary Co.

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LTD., Kizu, Kyoto. This blanching water was prepared by boiling 700 kg of bamboo shoots in 500ℓ water for 2 hours and stored in a freezer (1.4 kg bamboo shoot equivalents/1).

Preparations of the ethanol extract from the blanching water and of the *n*-butanol extract and the aqueous fraction from the ethanol extract were described in a previous paper[26]. From 13 1 blanching water, 130 ml of the ethanol extract was obtained (13 LE/130 ml). The concentrations of the ethanol extract and purified fractions were expressed in terms of liter equivalents to the blanching water per liter or milliliter (LE/1 or LE/ml). From 130 ml ethanol extract, 65 ml aqueous fraction (13 LE/65 ml) and 65 ml *n*-butanol extract (13 LE/65 ml) were obtained by partition with water-saturated *n*-butanol.

In the present study, the *n*-butanol extract was fractionated additionally by an ion-exchange Dowex 50W×8 (H^+, 200 mesh) column. The *n*-butanol extract (12 LE/60 ml) was diluted with 60 ml water and passed through a 2×22 cm column with Dowex 50W resin. The column was washed with 120 ml water and the washing was combined with the effluent. Substances held by Dowex 50W was eluted with 200 ml 3N NH_4OH. The effluent and the eluate were evaporated separately and dissolved each in 60 ml water (12 LE/60 ml). These two fractions were tested for their cytokinin activity and used for further experiments.

The eluate was developed in paper chromatography. One ml of the eluate (0.2 LE) was lined at 4 cm from the bottom of 40×40 cm Toyo filter paper No. 51 and the papers were developed ascendingly over 20 cm individually with four solvent systems, (A) water-saturated *n*-butanol, (B) ethylacetate : formic acid : water (60 : 5 : 35), (C) water-saturated *sec*-butanol and (D) 0.03 M boric acid. Chromatograms were cut crosswise into ten equal sections according to Rf value. Each section was cut into pieces and eluted with 25 ml of water twice in a steam bath. Each 50 ml eluate was added to 50 ml of culture medium in 2 times strength and tested for their cytokinin activity.

The eluate was treated further by silver ion precipitation. Ten ml of the eluate (2 LE/10 ml) was diluted to 40 ml and pH of the solution was adjusted to 1.0 with 1N H_2SO_4. Ten ml of $AgNO_3$ solution (0.2 g/ml) was added to the eluate, diluted with 80 ml water and stored overnight in a refrigerator. The sample was centrifuged and the supernatant was discarded. The precipitate was treated with 80 ml of 0.1N HCl, followed by addition of 80 ml of 80% ethanol and stirred at 50°C for 30 min. After the solution was centrifuged, the supernatant obtained was adjusted to pH 7.4 and evaporated *in vacuo*. The residue was dissolved in 20 ml water. The solution was layered with equal volume of water-saturated *n*-butanol three times. The *n*-butanol layer was evaporated *in vacuo* and the residue was dissolved in 20 ml water. The solution was designated as the Ag precipitate (2 LE/20 ml).

The Ag precipitate was fractionated by a Sephadex LH-20 column. The Ag precipitate (2 LE) in 20 ml was evaporated and the residue was dissolved in 2.5 ml 35% ethanol. The sample was poured into a 2×22 cm column of Sephadex LH-20 equilibrated in 35% ethanol and developed with 35% ethanol at 30 ml/hour. The eluate was collected by 5 ml. Each 1 ml from each fraction (0.4 LE) was tested after removing ethanol by the tobacco callus bioassay.

It has been known that olefinic double bond in N^6-side chain of adenine derivatives is destroyed by $KMnO_4$. In order to know whether the cytokinin in the active fraction obtained from Sephadex LH-20 fractionation is due to zeatin with an olefinic double bond in the side chain or to dihydrozeatin without it, the active fraction was treated with $KMnO_4$. The active fraction, 0.8 LE in 2 ml, was added with 0.01% $KMnO_4$ until permanganate color was kept for longer than several seconds. Then, the solution was kept in a room temperature for 15 minutes, and added with 5 ml ethanol to remove excessive $KMnO_4$. After evaporation the residue was dissolved in 20 ml water and the pH was adjusted to 7.4. The aqueous solution was layered with equal volume of water-saturated *n*-butanol three times. The aqueous layer was discarded. The *n*-butanol layer was evaporated, dissolved in 20 ml water and tested by the tobacco callus bioassay.

Bioassay procedures using tobacco callus (*Nicotiana tabacum* L. cv. Wisconsin No. 38) were carried out as described previously[27)28)]. The basal culture medium was the Linsmaier and Skoog medium[27)] containing the mineral salts, 30 g/l sucrose, 10 g/l agar, 100 mg/l *myo*-inositol, 2 mg/l IAA and 0.4 mg/l thiamine-HCl. All the samples to be tested were added to the basal media. After the agar was melted, the test media were distributed in aliquots of 20 ml in the 50 ml-conical flasks and autoclaved at 1.0 kg/cm^2 for 15 minutes. Three pieces of the tobacco callus (about 10 mg each in fresh weight) were implanted on the agar surface in each flask. Each experimental treatment contained 4 replicates of the flasks. The flasks were maintained at 28°C in the dark for 30 days, and then the fresh weight of tissues was determined.

Results and Discussion

The previous paper showed cytokinin activities of authentic zeatin and kinetin tested by the tobacco callus bioassay (Fig. 1)[26)]. Zeatin showed a detectable growth promotion at a very low concentration of 10^{-10}M. The callus yield increased with increasing concentrations and reached the maximum at 4×10^{-8}M. Higher concentrations reduced the growth promotion. Kinetin stimulated slightly the growth at 4×10^{-9}M, and the maximum callus yield was obtained at 10^{-7}M.

The cytokinin activity of the ethanol extract obtained from the blanching water of bamboo shoots was shown in Fig. 2 of the previous paper[26)]. The extract exhibited a slight cytokinin activity at 0.01 LE/l. The largest growth promotion was observed at 0.1 LE/l.

From the ethanol extract, the aqueous fraction and the *n*-butanol extract were obtained by partition with water-saturated *n*-butanol[26)]. As shown in Table 1 of Reference 26, the aqueous fraction showed a detectable cytokinin activity at a concentration of 0.1 LE/l and gave the maximum callus growth at 0.5 LE/l. On the other hand, the *n*-butanol extract gave the maximum callus yield at 0.1 LE/l and higher concentrations rather reduced the cytokinin activity, indicating coexistence of some inhibitory substances.

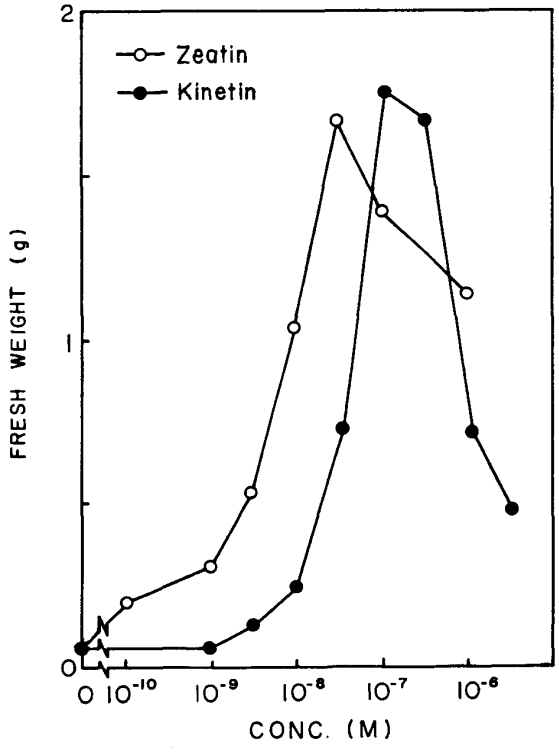

Fig. 1. Effects of zeatin and kinetin on the growth of tobacco callus *in vitro*.

The previous paper pointed out that the cytokinin in the aqueous fraction was likely to be zeatin ribotide and that about 2×10^{-5} mole of this cytokinin was contained in the aqueous fraction equivalent to 1 liter of the blanching water.

In the present study, cytokinins in the *n*-butanol extract were further fractionated into the effluent and the NH_4OH eluate by a column of Dowex 50W×8 (H^+) resin and tested for their cytokinin activity. As shown in Table 1, the effluect was nearly inactive at 0.01-0.5 LE/l, while the eluate was weakly stimulative at 0.01 LE/l. Higher concentrations gave stronger cytokinin activity with the maximum callus yield at 0.5 LE/l. From Fig. 1, it was calculated that the cytokinin activity of the eluate at the concentration of 0.5 LE/l corresponded approximately to that of 20 nM zeatin.

Cytokinins in the eluate were studied further by paper chromatography. On chromatogram developed with water-saturated *n*-butanol, a strong activity located at Rf 0.7-0.8, where authentic zeatin moved with (Fig. 2A). At the position of authentic zeatin riboside, a small cytokinin activity was observed. At Rf 0.1-0.2, another small activity was observed indicating coexistence of a cytokinin more soluble in water than zeatin. N^6-(Δ^2-Isopentenyl)adenine and its riboside located around Rf 0.9, where no cytokinin activity was found. Chromatogram developed with ethylacetate : formic acid : water (60 : 5 : 35) showed the main cytokinin activity at Rf 0.1-0.2 where zeatin and its riboside were located (Fig. 2B). However, at the positions of N^6-(Δ^2-isopentenyl)adenine and its riboside any significant cytokinin activity was not observed again. In the case of chromatogram developed with water-saturated *sce*-butanol (Fig. 2C), two peaks of the cytokinin activity were observed; one large peak was at Rf 0.7-0.8, where zeatin located and another small activity at Rf 0.5-0.6. The Rf of zeatin riboside seemed to be different from this small activity. On chromatogram developed with boric acid, a broad activity locating between Rf 0.5 and 0.8 was observed (Fig. 2D). The Rf of this peak contained that of authentic zeatin, but seemed to be different from that

Table 1. Cytokinin activity of the effluent and the eluate after application to a Dowex 50W×8 column.

Additives	Conc. (LE/1)*	Fresh weight of callus (g)
—	0	0.06
Effluent	0.01	0.05
	0.05	0.08
	0.1	0.05
	0.5	0.04
Eluate	0.01	0.09
	0.05	0.28
	0.1	0.32
	0.5	1.35

* see Materials and Methods.

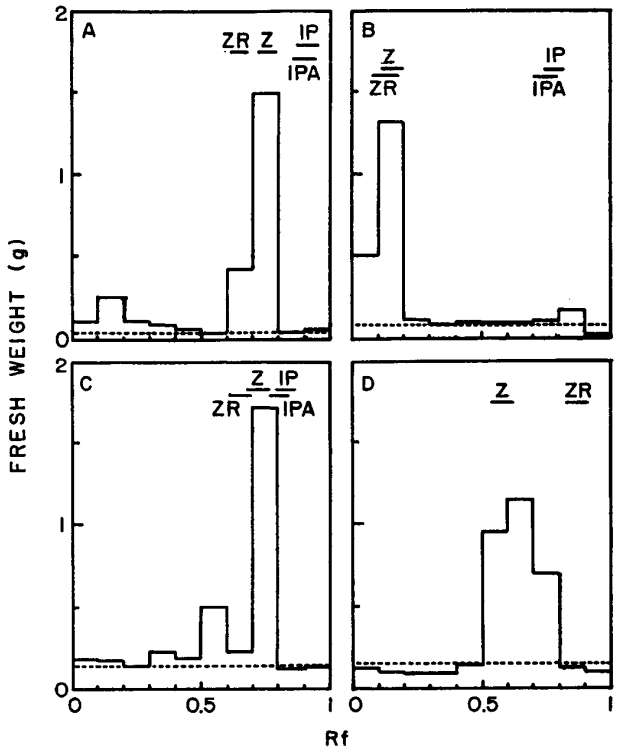

Fig. 2. Location of cytokinin activity in paper chromatograms of the Dowex 50W eluate developed with (A) water-saturated *n*-butanol, (B) ethylacetate : formic acid : water (60 : 5 : 35), (C) water-saturated *sec*-butanol and (D) 0.03 M boric acid. Lines at the top of histograms indicate the loci of zeatin (Z), zeatin riboside (ZR), N^6-(Δ^2-isopentenyl)-adenine (IP) and N^6-(Δ^2-isopentenyl)adenosine (IPA) co-chromatographed. The broken lines represent the fresh weight of the controls without cytokinin.

of zeatin riboside. Thus, these chromatograms indicate that the main cytokinin activity of the Dowex 50W eluate is caused by zeatin, but cytokinin activity due to zeatin riboside is not clear, although a small activity seems to be due to this riboside, and that neither N^6-(Δ^2-isopentenyl)adenine nor its riboside was contained in the blanching water of bamboo shoots.

Cytokinins in the Dowex 50W eluate was purified additionally by silver ion precipitation. As shown in Table 2, Ag precipitate gave a weak cytokinin activity at 0.01 LE/l. With increasing concentration the cytokinin activity increased. The cytokinin activity of Ag precipitate equivalent to 1 liter of the blanching water corresponded to that of 2×10^{-8}mole of zeatin. A separate experiment revealed that the fraction which was not precipitated by silver ions exhibited no cytokinin activity.

The Ag precipitate of 1 LE was developed again in paper chromatography with two solvent systems, water-saturated *n*-butanol and 0.03M boric acid. Location of cytokinin activity on paper chromatogram developed with water-saturated *n*-butanol was shown in Fig. 3. The main activity was observed at Rf 0.6–0.8. In this zone zeatin and zeatin riboside located with Rf 0.77 and 0.66 respectively. Location of cytokinin activity on paper chromatogram developed with 0.03M boric acid, although not presented as Figure, resembled Fig. 2D. These results indicate that Ag precipitation did not cause chemical conversion of the cytokinin in the Dowex 50W eluate and that the main cytokinin activity of Ag precipitate could ascribe to zeatin.

The Ag precipitate was further fractionated in a column of Sephadex LH-20 with 35% ethanol. As shown in Fig. 4, the elution volume of the main cytokinin activity corresponded to that of zeatin. Nevertheless, no cytokinin activity corresponding to zeatin riboside was found.

Generally, it has been known that dihydrozeatin which is hydroxylated in N^6-side chain of zeatin moved closely with zeatin in not a few paper chromatographies even using various kinds of solvent systems. Therefore, it is indispensable to check whether the main cytokinin activity of the Ag precipitate is due to zeatin or dihydrozeatin. $KMnO_4$ has been known to destroy olefinic double bond in N^6-side chain of adenine derivatives such as zeatin, but not to destroy dihydrozeatin without double bond in N^6-side chain. The Ag precipitate was treated with $KMnO_4$ and its cytokinin activity was tested. Table 3 indicates that the Ag precipitate treated with $KMnO_4$ lowered cytokinin activity. A separate

Table 2. Cytokinin activity of the Ag precipitate tested by the tobacco callus bioassay.

Additives	Conc. (LE/1)*	Fresh weight of callus (g)
—	0	0.05
Ag precipitate	0.01	0.12
	0.05	0.22
	0.1	0.35
	0.5	0.98
	1	0.99
	5	1.40

* see Materials and Methods.

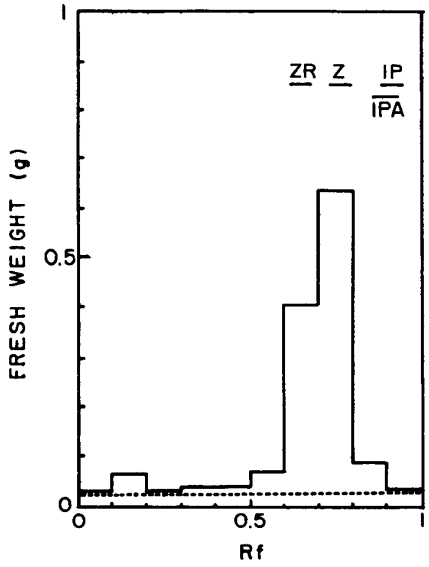

Fig. 3. Location of cytokinin activity on paper chromatogram of Ag precipitate developed with water-saturated *n*-butanol. Lines at the top of histogram indicate the loci of zeatin (Z), zeatin riboside (ZR), N^6-(Δ^2-isopentenyl)-adenine (IP) and N^6-(Δ^2-isopentenyl)adenosine (IPA) co-chromatographed. The broken line represents the fresh weight of the control without cytokinin.

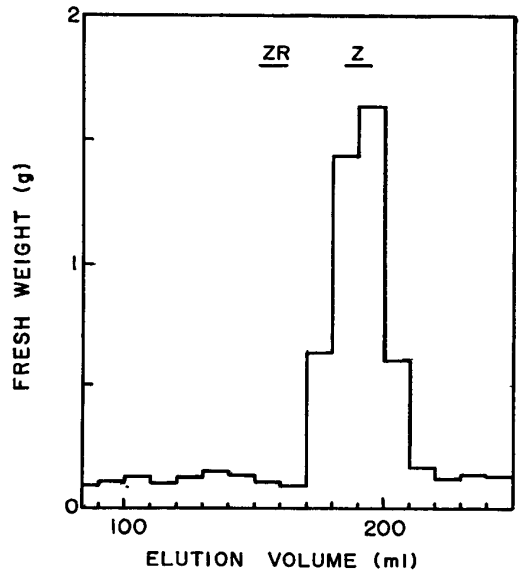

Fig. 4. Cytokinin activity in Ag precipitate after fractionation on a Sephadex LH-20 column developed with 35% ethanol. Lines at the top of histogram indicate the loci of zeatin (Z) and zeatin riboside (ZR) co-chromatographed.

Table 3. Cytokinin activity of Ag precipitates with and without $KMnO_4$ treatment.

Additives	Conc. (LE/1)*	Fresh weight of callus (g)
—	0	0.03
Ag precipitate	0.08	0.44
	0.72	0.83
$KMnO_4$-treated Ag precipitate	0.08	0.11
	0.72	0.14

* see Materials and Methods.

attempt revealed that the cytokinin activity of dihydrozeatin was not reduced by $KMnO_4$ treatment.

Thus, all these results obtained in the present study support a tentative conclusion that the water-unsoluble cytokinin present in the blanching water of bamboo shoots is likely to be zeatin, and that a comparison between the cytokinin activity of authentic zeatin and that of the Ag precipitate indicates presence of about 2×10^{-8} mole of zeatin in 1 liter of the blanching water.

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